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BINDING OF NEW METHYLENE BLUE TO ENDOTOXINS AND ITS EFFECTS ON THE ENDOTOXIN ACTIVITY STUDIED BY DOUBLE DIFFUSION AND LIMULUS AMEBOCYTE LYSATE ASSAYS

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New methylene blue was found to bind to smooth and rough forms of endotoxins and their components lipid A and lipid X in agarose double diffusion experiments. It also bound to dextran sulfate, heparin, transfer RNA, succinylated bovine serum albumin, trypsin, pepsin and casein, but not bovine serum albumin. Charge-charge interactions between the dye and these molecules to the formation of dye-endotoxin complex. Binding of the dye to endotoxin was found to block the endotoxin function in both gelation and chromogenic limulus amebocyte lysate assays. The potency of the dye in such inhibition was comparable to that of polymyxin B.

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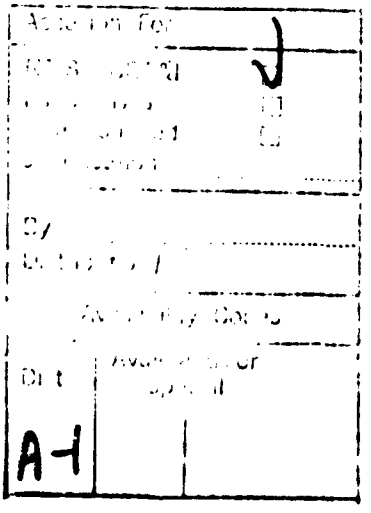

INTRODUCTION

Endotoxin (or lipopolysaccharide, LPS) exists ubiquitously on the outer membrane of Gram-negative bacteria. Its structure (Figure 1-I) contains O-antigen polysaccharide, core polysaccharide and lipid A (Rietschel et al., 1984; Luderitz et al., 1982). The O-antigen polysaccharide is composed of repeating oligosaccharide, specific to the species and the strain of the bacteria; the core polysaccharide consists of 11 or less monosaccharide units including three 2-keto-3-deoxyoctonate (KDO), and is more conserved structurally than the O-antigen polysaccharide; lipid A, which is the most conserved portion in the LPS of different origins, has two glucosamine units attached with six or more fatty acid chains and two phosphate groups (Rietschel et al., 1984; Luderitz et al., 1982). The lipid A moiety is embedded in the bacterial membrane while the polysaccharide chain is extruding out of the membrane into hydrophilic surroundings. When LPS is released from bacteria into the medium, it forms micelles (in the shape of ribbons, disks and lamellae, etc.) and vesicles of bilayer structure (Figure 1-II; Shands and Graham, 1969; Rothfield and Horne, 1967; Shands et al., 1967; Beer et al., 1966; Work et al., 1966) with the hydrophobic portion (fatty acid chains) buried and the hydrophilic portion (the phosphate groups, KDO and the polysaccharide chain) exposed to the aqueous medium (Figure 1-II).

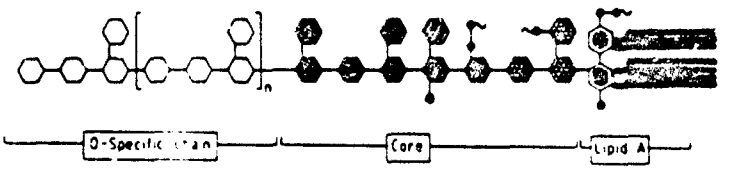

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FIGURE 1-I

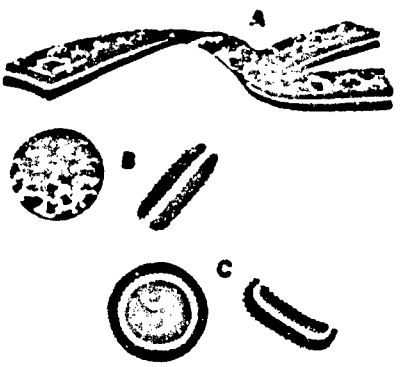

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FIGURE 1-II

Endotoxemia accompanies infections with Gram-negative bacteria and has been shown by several investigators to be associated with the morbidity and mortality (60%) in septic shock (Cohen et al., 1987; Rietschel et al., 1982; Shine et al., 1980). The structural diversity of LPS (e.g. the polysaccharide chain) from different origins and its amphipathic property have posed serious difficulties to the prospect of its broad spectrum recognition by immunoglobulins that constitute a critical immunologic defense mechanism among others (Lachman et al., 1984; Ziegler et al., 1982; Gaffin et al., 1981). Polymyxin B, a cyclic polypeptide antibiotic, has been found to bind to all LPS (Cohen et al., 1987; Issekutz, 1983; Morrison and Jacobs, 1976), and had been used as a potent anti-endotoxin remedy (Craig et al., 1974; Corrigan and Bell, 1971; Rifkind, 1967). Its systemic application, however, is limited by the accompanying neurotoxicity and nephrotoxicity (Nord and Hoeprich, 1964; Wolinsky and Hines, 1962). Recently, several LPS-binding proteins have been identified and investigated (Tobias et al., 1988; 1986; Nakamura et al., 1986; Aketagawa et al., 1986; Brade and Brade, 1985; Liang et al., 1980; Johnson et al., 1977). In this paper, we report on the binding of new methylene blue (Figure 2) to endotoxin. Its effects on the function of LPS in both gelation and chromogenic limulus amebocyte lysate (LAL) assays (Novitsky et al., 1982; Iwanaga et al., 1978; Levine and Bang, 1968; 1964) are evaluated.

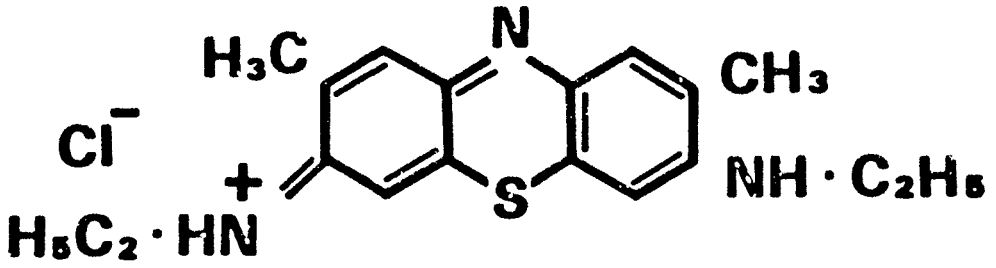

FIGURE 2

MATERIALS AND METHODS

Endotoxins, lipid A, lipid X and polymyxin B were purchased from Sigma Chemical Company, Calbiochem, List Biochemicals or Ribi Immunochemicals. Standard endotoxin EC-5 (Hochstein et al., 1983; Rudbach et al., 1976) and smooth LPS of *N. meningitidis* were kindly provided by Drs. D. Hochstein and M.-C. Tsai (U.S. Food and Drug Administration), respectively. New methylene blue was obtained from Kodak Chemicals. Dextran sulfate, heparin, transfer RNA (tRNA) were respectively purchased from Pharmacia, Fisher and Boehringer Mannheim. Succinylated bovine serum albumin (SuBSA) was prepared by succinylation of BSA with succinyl anhydride (Klotz, 1967). The chromogenic LAL assay kit was obtained from Whittaker Biochemicals; and the gelation LAL test kit was from Sigma Chemical Company.

Double diffusion (DD) assay

Agarose (0.5%, 1.5 mm thick) in 2.5 mM EDTA and 5 mM phosphate buffer, pH 7.4, was prepared in leveled petri dishes. Wells of 4.5 mm diameter were punched and loaded with 8-15 ul samples (0.3-2 mg/ml for the test molecules and the dye). The plates were examined for formation of precipitation bands after 7 hours or overnight incubation in a sealed, moist chamber at room temperature. For the purpose of eliminating the charge-charge interactions, some DD experiments were carried out in plates containing additional 3.4 M CsCl.

Gelation limulus amebocyte lysate (LAL) assay

Following the procedure provided by the supplier (Sigma Chemical Company), 100 ul of the sample (EC-5 LPS or its mixture with dye) was mixed with 100 ul of LAL lysate in a 5 ml sterile, capped polypropylene tube and then was incubated in a 37°C dry incubator without disturbance for an hour. The result was scored by observation of the fluidity of the mixture. A positive LAL test was indicated by the gelation of the mixture, which stayed at the bottom of the gently inverted tube.

Chromogenic LAL assay

The LAL assay was conducted primarily according to the protocol provided by the manufacturer (Whittaker Biochemicals) except for some minor modifications. The assay was carried out with sterile, flat-bottomed 96-well tissue plate with lid. 40 ul sample (LPS EC-5 or its mixtures with dye or polymyxin B) was loaded to the well followed by the addition of 25 ul of limulus amebocyte lysate. The plate, with the lid, was then incubated in a 37°C water bath for 10 minutes followed by the addition of 50 ul of substrate. The plate was further incubated in the bath for 12 minutes, and then 50 ul of 25% acetic acid was added to stop the reaction. The extent of the reaction was monitored by a Dynatech plate reader at 405 nm.

RESULTS

Aggregation of new methylene blue in aqueous solution

In order to understand the state of new methylene blue molecule in aqueous solution, the concentration dependency of its absorption at 634 nm was studied. Linear relationship existed at 4 ug/ml or lower (Figure 3). This indicated that the dye aggregated at concentrations higher than 4 ug/ml.

Binding of new methylene blue to endotoxins by double diffusion

The interactions between new methylene blue and LPS, lipid A or lipid X were investigated by the methods of double diffusion in agarose gel. Figure 4 shows the typical precipitation bands formed by the dye and LPS or its components. All the endotoxins (Table I) tested in this study showed precipitation band. Thus, the binding of the dye to endotoxin, like that of polymyxin (Morrison and Jacobs, 1976; Issekutz, 1983; Cohen et al., 1987) and LPS-binding proteins (Tobias et al., 1988; 1986; Nakamura et al., 1986; Aketagawa et al., 1986; Brade and Brade, 1985; Liang et al., 1980; Johnson et al., 1977), is common to all the LPS.

Binding of new methylene blue to molecules containing multiple anionic groups

In order to understand the nature of the dye binding, various molecular species were tried out for their possible interactions with the dye. It was found that only those molecules containing multiple groups of negative charge were able to exhibit binding

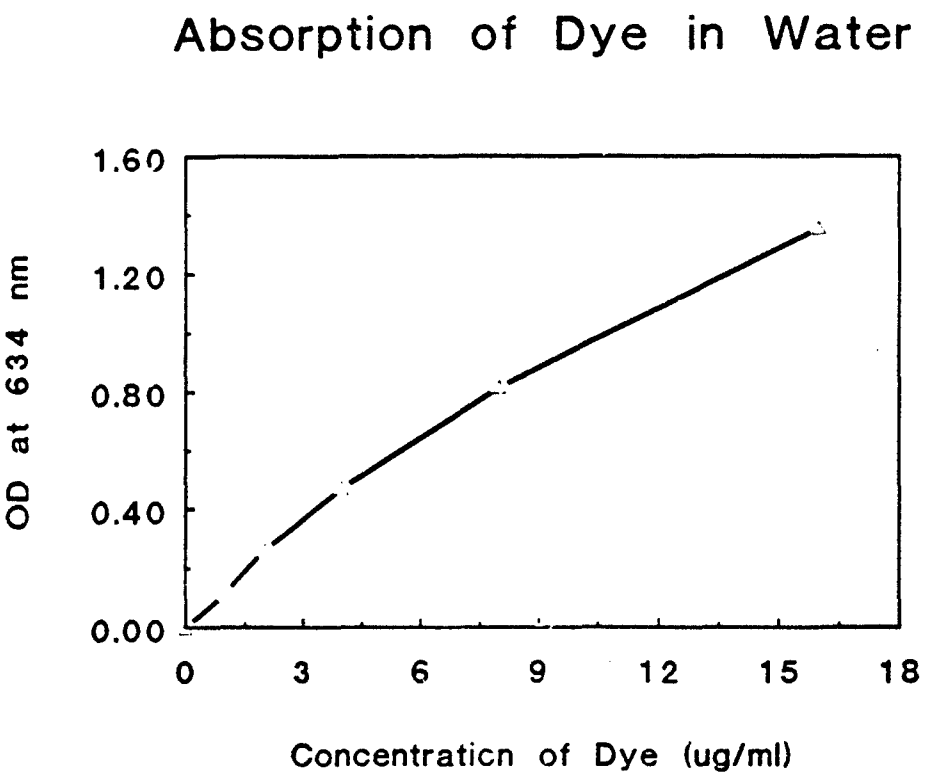

FIGURE 3

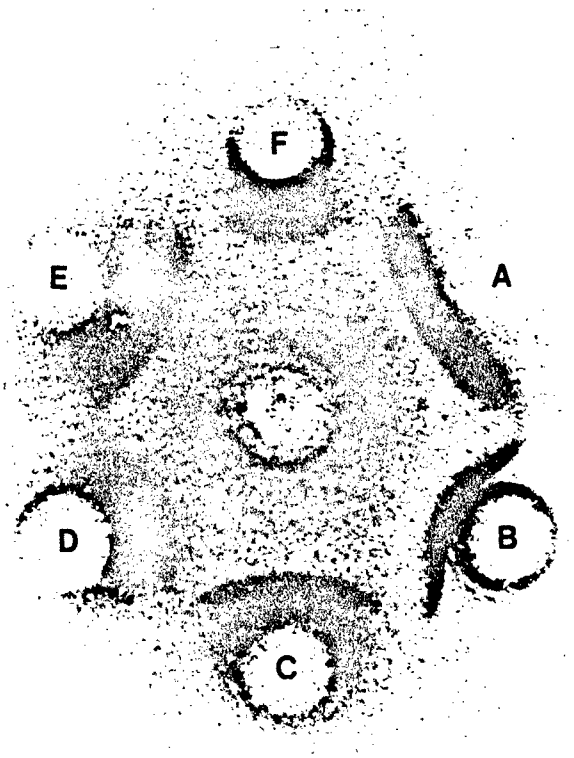

FIGURE 4

Table I. Endotoxins tested against new methylene blue in agarose double diffusion experiments.*

Smooth LPS	Rough LPS	Lipid A	Lipid X
Bor. pertussis	E. coli 0111B4 (J5, Rc)	E. coli K12	E. coli
E. coli 055B5	E. coli K12 (Re)	E. coli 0111B4	
E. coli 0111B4	Sal. minnesota (Re)	Sal. minnesota	
E. coli 0127B8	Sal. minnesota (Rd)		
E. coli 0128B12	Sal. minnesota (Rc)		
K. pneumonia	Sal. minnesota (Rb)		
N. meningitidis	Sal. minnesota (Ra)		
Sal. abortus equi			
Sal. enteritidis			
Sal. minnesota			
Sal. typhimurium			
Ser. marcescense			
V. cholerae			
Y. enterocolitica			

* All the endotoxins formed precipitation band with the dye.

to the dye under the experimental condition. For example, bovine serum albumin did not react with the dye, while its succinylated derivative did (Figure 5-I). Other molecules interacting with the dye included dextran sulfate, heparin, tRNA (or polynucleotides), trypsin, pepsin and casein (Fig. 5-I and Table II). This indicated that the binding of the dye, which has a positive charge delocalized between two amino groups through polyaromatic structure, is via charge-charge interactions.

Inhibition of the dye binding by 3.4 M CsCl

To further confirm the role of the charge-charge interactions in the dye binding, double diffusion experiments were carried out in the presence of 3.4 M CsCl. Under this condition, such interactions should be eliminated (Morrison and Jacobs, 1976). No precipitation band was observed for dextran sulphate, heparin, SuBSA and tRNA. (Figure 5-II) In contrast, visible precipitation band still showed with smooth LPS of E. coli O111B4, even though the band was not as intense as those in agarose plates lacking CsCl (Figure 5-I). This suggested that hydrophobic interactions were also involved in the dye-LPS complex formation.

Inhibition of LAL assays by new methylene blue

Activation of some component (or components) in the limulus amebocyte lysate (LAL) by endotoxin leads to gelation of the lysate or release of chromogenic molecule from the added synthetic substrate. LAL assay has been widely used for

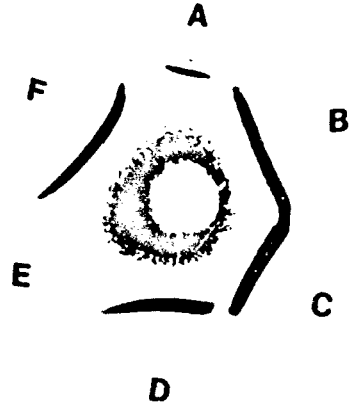

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FIGURE 5-I

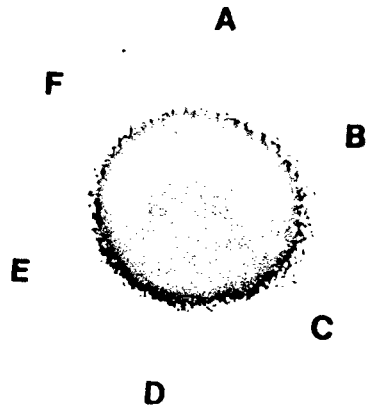

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FIGURE 5-II

Table II. Molecular species that precipitated with new methylene blue in agarose double diffusion experiment.

Protein	**Carbohydrate**	**Polynucleotide**
casein	dextran sulfate	transfer RNA
pepsin	heparin	
trypsin		
succinylated BSA		

detection and quantitation of LPS (Jorgensen, 1986). Data in Table III indicated that both new methylene blue and polymyxin B inhibited gelation of LAL caused by LPS. However, the LPS-blocking activity of polymyxin B was reduced after prolonged incubation (4 hr or longer) of the mixture (data not shown). This might be attributed to digestion of polymyxin B by the proteolytic enzymes in the LAL.

Binding of new methylene blue or polymyxin B to endotoxin was found to block LPS function in the chromogenic LAL test, too (Figures 6-I and II). The potency of the dye, under the experimental condition, was comparable to that of polymyxin B. For 50% inhibition of 25 pg/ml EC-5 LPS, 7 ug/ml dye or 0.5 ug/ml polymyxin B was needed. In the case of 100 pg/ml LPS, 14 ug/ml dye or 1.2 ug/ml polymyxin B was required to achieve 50% inhibition.

Table III. Inactivation of LAL gelation activity* of EC-5 LPS by new methylene blue and polymyxin B.

	Concentrations of added EC-5 (ng/ml)					
	80	8	0.8	0.08	0.008	0
LAL + LPS	+	+	+	+	-	-
LAL + LPS + dye (300 ug/ml)	+	-	-	-	-	-
LAL + LPS + polymyxin B (200 ug/ml)	+	+	-	-	-	-

* Evaluation of LAL assay: +, gelation; -, no reaction.

I. LAL Activity in Dye Solution

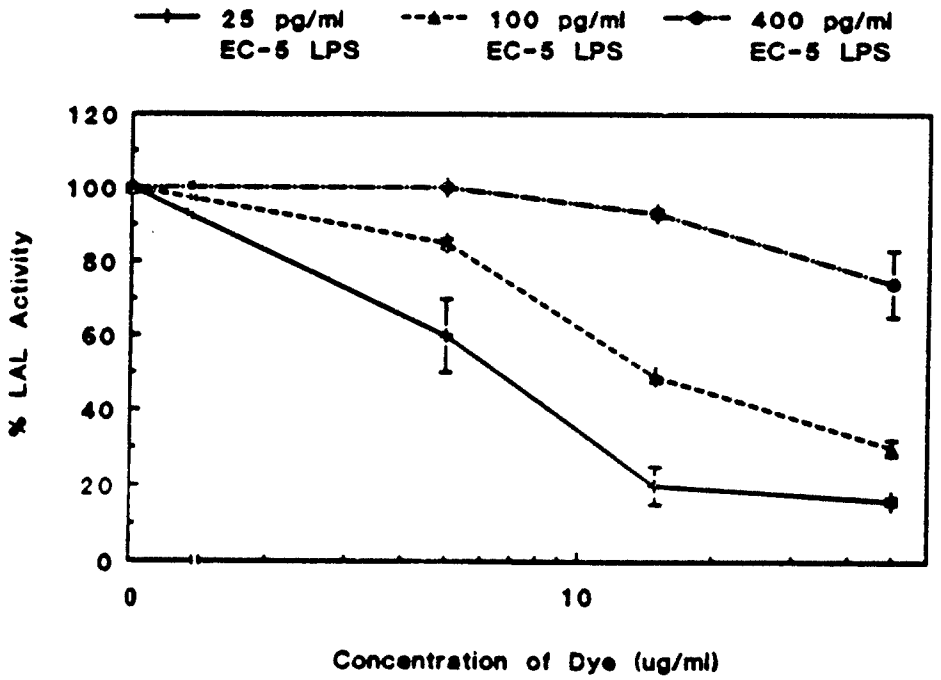

FIGURE 6-I

II. LAL Activity in Polymyxin B Soln.

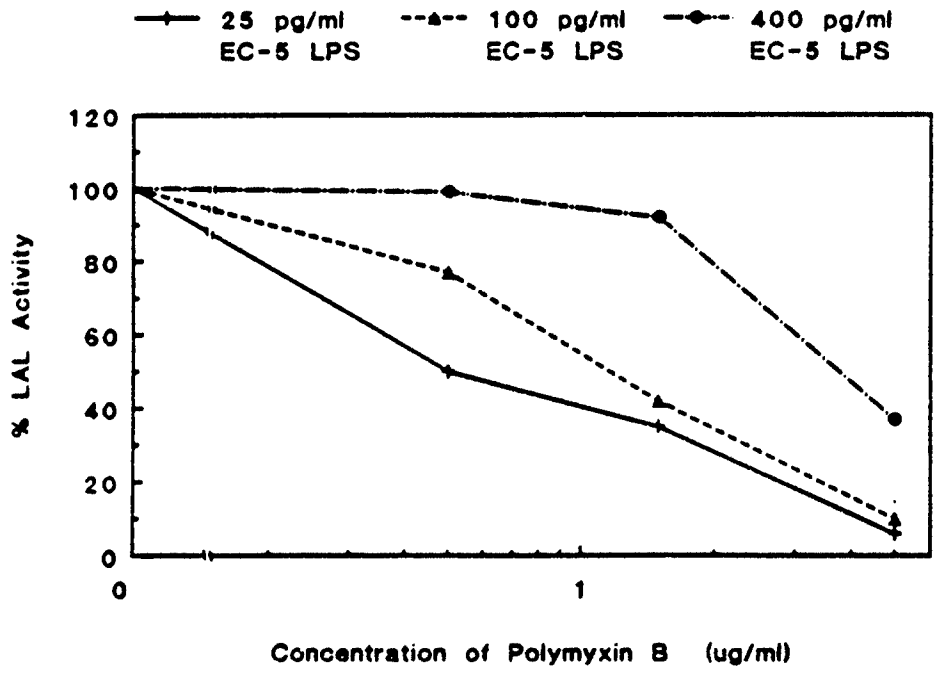

FIGURE 6-II

DISCUSSION

The binding of new methylene blue to endotoxin (Figure 4 and Table I) is primarily due to charge-charge interactions and secondly, hydrophobic interactions. Because of the amphipathic property of its structure, endotoxin exists in bilayer structure as micelles and/or vesicles (Figure 1-II) in aqueous solution (Shands and Graham, 1969; Rothfield and Horne, 1967; Shands et al., 1967; Work et al., 1966; Beer et al., 1966). Such aggregates of 'ordered' structure prevail for all LPS in solution despite the structural diversity in O-antigen and core polysaccharides. On the surface of the micelles and the vesicles exists high density of negative charge from phosphate groups on lipid A and core polysaccharide, and from the carboxylic groups of KDO in core polysaccharide (e.g. in Salmonella LPS, 11 negative charges and 3 positively charged ethanolamine groups are known; Figure 1-I). Such polyanionic charge is a common structural characteristic of the LPS, exposing to the hydrophilic environments. On the other hand, the structure of new methylene blue contains a positive charge, which is delocalized between the two amino groups through the aromatic ring system (Figure 2). Thus, each dye molecule is a bifunctional species for the binding process. Moreover, the optical density of the dye indicated that it was in aggregated state at concentrations higher than 4 ug/ml (Figure 3) with multiple positive charges exposed to the hydrophilic surroundings. This will enhance its charge-charge interactions with the polyanionic micelles and/or vesicles of

endotoxin.

Once charge-charge interactions occur, conformational changes may be induced inside the aggregates of both LPS and new methylene blue such that hydrophobic interactions between the aromatic ring of the dye and the fatty acid chains of the LPS may be exerted to enforce the complexation.

The fact that new methylene blue also bound to other molecules such as dextran sulfate, heparin, SuBSA, tRNA, but not BSA (Figure 5-I), provided additional evidence for the requirement of the presence of multiple negatively charged groups on the bound molecules. Proteins such as trypsin, pepsin and casein were found to be in this category (Table II). The role of the charge-charge interactions in the dye binding were further substantiated by the result from the experiment in the presence of 3.4 M CsCl (Figure 5-II). Under this condition, such interactions were deprived, and precipitation bands for dextran sulfate, heparin, SuBSA and tRNA were not observed. Visible precipitation band, however, continued to show with endotoxin samples even though weak when it was compared with those in plates lacking CsCl. This is indicative of the involvement of hydrophobic interactions in the dye-LPS complex formation.

Inhibition of the gelation and chromogenic LAL assays by the dye and polymyxin B further suggested that both bound to the LPS at or nearby its functional site, which is close to the negatively charged groups, i.e. phosphate, KDO and others. Data in Fig. 6-I and II indicated that more dye (10 times in mass)

than polymyxin B was needed to block the same level of endotoxin function in LAL assay. Polymyxin B has molecular weight of 1200 with 5 positive charges, giving 240 equivalent weight per charge in comparison to 360 for the dye. With this consideration, the potency of polymyxin B in inhibiting LPS function become 7 times that of the dye. This may be attributed to the difference between the detailed geometries of the two molecules. When the LPS concentration increased four times, the dye only needed to increase two times in order to achieve 50% inhibition. This is in line with the observation that the dye aggregated in aqueous solution at concentration higher than 4 ug/ml, and that aggregation of the dye enhanced its interactions with endotoxin. Similar phenomenon was observed for polymyxin B.

CONCLUSION

We have found that new methylene blue binds to endotoxin. The binding is primarily due to charge-charge interactions between the aggregated dye and the bilayered LPS micelles and/or vesicles. Hydrophobic interactions are also involved. The binding appears to be universal to all the LPS of different origins, and is further extended to polyanionic species such as dextran sulfate, heparin, succinylated BSA, tRNA, trypsin, pepsin and casein. New methylene blue was also shown to inhibit the function of LPS in gelation and chromogenic LAL assays with one seventh potency of polymyxin B under the present experimental conditions.

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FIGURE LEGENDS

Figure 1. Structure of endotoxin (1-I), taken from Rietschel et al., 1984; and schemes of its aggregated forms, micelles and vesicles (1-II) taken from Shands and Graham, 1969.

Figure 2. Structure of new methylene blue.

Figure 3. Concentration dependency of absorption of new methylene blue in aqueous solution at 634 nm.

Figure 4. Reactions of new methylene blue and some endotoxins in agarose double diffusion plate after overnight incubation. The amount of the dye (center well) used is 12 ug, and that of the endotoxins (peripheral wells) is 30 ug. The endotoxins tested are lipid A of E. coli, (A); rough LPS of E. coli O111B4 or J5, (B); smooth LPS of E. coli O111B4, (C); smooth LPS of Salmonella typhimurium, (D); smooth LPS of Serratia marcenscens (E) and smooth LPS of Klebsiella pneumonia, (F).

Figure 5. Reactions of new methylene blue (12 ug, center well) with other molecules (peripheral wells) in agarose double diffusion plates. The doses of these molecules are (A) 30 ug, smooth LPS of E. coli O111B4; (B) 5 ug, dextran sulfate; (C) 10 ug, heparin; (D) 5 ug, succinylated BSA; (E) 5 ug, BSA; (F) 18 ug, tRNA (Fig. 5-I). Similar experiment was carried out in the presence of additional 3.4 M CsCl (Fig. 5-II) where the design of

the wells and the doses of the samples are the same as those in (Fig. 5-I). The plates shown were after 7 hours incubation; however, the pattern of the precipitation bands remained essentially unchanged after overnight incubation.

Figure 6. Inhibition of chromogenic LAL assay by new methylene blue (Fig. 6-I) and polymyxin B (Fig. 6-II). The experiments were carried out with three concentrations (i. e. 25, 100 and 400 pg/ml) of standard EC-5 LPS. The results were expressed as % LAL activity against the doses (in log scale) of new methylene blue or polymyxin B in the system.